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Head Clerk

GLUCOSE DEPENDENT RELEASE OF INSULIN FROM GLUCOSE SENSING INSULIN DERIVATIVES

Field of the invention

The present invention relates to insulin derivatives having a built-in glucose sensor, capable to deliver insulin from a depot as a function of the glucose concentration in the surrounding medium (*e.g.* tissue). In one embodiment of the invention, the insulin derivatives having a built-in glucose sensor are integrated in protracted acting, water-soluble aggregates of the derivatives in which the propensity to aggregation diminishes, and thereby the rate of absorption of the insulin is increased, as the concentration of glucose in the surrounding medium (*e.g.* tissue) is increased. In another embodiment of the invention, crystalline compositions of insulin derivatives having a built-in glucose sensor are provided. If the concentration of glucose in the surrounding medium (*e.g.* tissue) is increased, the rate of dissolution of the insulin crystals is enhanced, and hence the rate of absorption increases. The invention relates to insulin derivatives having a built-in glucose sensor, to pharmaceutical compositions comprising such insulin derivatives capable of releasing insulin as a function of the glucose concentration, and to the use of such compositions in the treatment of diabetes.

Background of the invention

Diabetes is a general term for disorders in man having excessive urine excretion as in diabetes mellitus and diabetes insipidus. Diabetes mellitus is a metabolic disorder in which the ability to utilize glucose is partly or completely lost.

Since the discovery of insulin in the 1920's, continuous strides have been made to improve the treatment of diabetes mellitus. To help avoid extreme glycaemia levels, diabetic patients often practice multiple injection therapy, whereby insulin is administered with each meal. Many diabetic patients are treated with multiple daily insulin injections in a regimen comprising one or two daily injections of a protracted insulin composition to cover the basal requirement, supplemented by bolus injections of a rapid acting insulin to cover the meal-related requirements.

Protracted insulin compositions are well known in the art. Thus, one main type of protracted insulin compositions comprises injectable aqueous suspensions of insulin crystals or

amorphous insulin. In these compositions, the insulin compounds utilised typically are protamine insulin, zinc insulin or protamine zinc insulin.

When human or animal insulin is brought to form higher associated forms, *e.g.* in the presence of Zn^{2+}-ions, precipitation in the form of crystals or amorphous product is the result; see for example pages 20-27 in Jens Brange, *Galenics of Insulin*, Springer Verlag (1987). Thus, at pH 7, addition of 6 Zn^{2+} ions per insulin hexamer to a solution of porcine insulin will lead to an almost complete precipitation of the insulin.

Another type of protracted insulin compositions is solutions having a pH value below physiological pH from which the insulin analogue will precipitate because of the rise in the pH value when the solution has been injected. This principle may be combined with the present invention by incorporation of the glucose-sensor in the insulin analogue. In addition to the glucose sensor these analogues have mutated asparagine in position A21 to an acid-stabile residue such as glycine or serine or alanine and have mutations to increase the net charge of the molecule by about 2, *e.g.* by B27 Thr -> Arg and B30 Thr-OH -> Thr-NH_2 mutations or by addition of basic residues, *e.g.* B31-B32 Arg-Arg.

Soluble insulin derivatives containing a lipophilic substituent linked to the ε-amino group of a lysine residue in any of the positions B26 to B30 have been described in the literature. Such derivatives have a protracted action after subcutaneous injection as compared to soluble human insulin, and this protracted action has been explained by a reversible binding to albumin in subcutis, blood and peripheral tissue.

An additional mechanism of prolonging the action of some of the soluble insulin derivatives featuring a lipophilic substituent has been disclosed, i.e. derivatives capable of forming high molecular weight aggregates, being larger than aldolase (Mw = 158 kDa) when analysed in a defined gel filtration system.

In normal persons blood glucose concentration is about 5 mM, rising to about 7 mM after the meals. To day, even when applying the most advanced and incentive insulin treatment, using rapid acting insulin analogues for meal-related injections and soluble depot insulin for basal insulin based on frequent monitoring of blood glucose, diabetics often experience glucose concentrations out of control. If to much insulin is administered, so that glucose concentrations get below about 3 mM, hypoglycaemic events might occur, leading to unconsciousness. When too little insulin is administered and glucose concentrations rises to about 20 mM acetone appears in the blood and give rise to diabetic ketoacidosis and, eventually, diabetic coma. However, it is desirably to control the blood glucose concentration of diabetics more tightly, as close to the 5 mM as possible, in order to diminish diabetic late complications. The DCCT (Diabetes Complication

Clinical Trial) study from 1993 in USA examined the development of diabetic complications in type 1 diabetics during 9 years (N Engl J Med 1993, 329, 977-986). The UKPDS (United Kingdom Prospective Diabetes Study) studied the development of complications in type 2 diabetics during 15 years (Lancet 1998, 352, 854-865). Even though the pattern of complications differs between these two types of diabetics both investigations conclude that a tight control of blood glucose results in a marked reduction of complications. Thus, there is an unmet medical need for means to obtain glucose control in diabetics closer to the normal value of 5 mM.

In theory, one way to obtain tight glucose control would be to couple a glucose sensor, positioned in the tissue of the patient, to a computer that controls an insulin pump. The pump is via a catheter connected to a needle inserted under the skin. However, it appears as if such a feed back control system has not yet been implemented, possible because of lack of stable and reliable of glucose sensors. Glucose sensors inserted in the tissue appears to get overgrown with fibrin, and it appears that non-invasive sensors, *e.g.* based on infrared optics, remain to be invented or developed.

Attempts to develop systems for glucose dependent release of insulin from a depot has previously been described. A carbohydrate binding lectin, such as concanavalin A, immobilized to a solid matrix, such as hollow fibres, binds an insulin derivative substituted with a carbohydrate moiety, such as maltotriose, maltose or dextran. The matrix allows diffusion of glucose and insulin derivative. As the systemic glucose concentration rise glucose displaces increasing amounts of the insulin derivative from the matrix, thus making more insulin available to the circulation, and subsequently to the insulin receptors, when it is needed. It appears as if none of these lectin based systems have been implemented clinically, probably due to the inconvenience of implantating the insulin containing matrix in the body, and to the danger of carrying a large insulin depot within the body.

Another concept to a glucose-controlled insulin release system is based on the glucose oxidase catalysed conversion of glucose to gluconic acid. The glucose oxidase is immobilized to a matrix, *e.g.* of ethylene/vinyl acetate copolymer, and the insulin or insulin derivative is trapped in the matrix in the solid state. As the pH is lowered locally due to the production of gluconic acid the solubility of insulin increases. Thus, the rate of release of soluble insulin from the solid state reflects the glucose concentration. Likewise, it appears as if none of these glucose oxidase based systems have been implemented clinically, possibly for the same reasons.

Furthermore, attempts to provide glucose controlled insulin release from a depot, in which the glucose sensing molecular structure is part of a matrix, i.e. a soluble or solid polymer have been made.

Summary of the invention

We have invented a new insulin derivatives from which the release of insulin from an injected or inhaled depot thereof is glucose dependent. In the depot the insulin derivative, modified with a glucose sensor, is either in the crystalline state or in a highly aggregated soluble state, states which bring about the protracted absorption from the site of injection. The solubility of the crystals and the state of aggregation in the soluble aggregates are influenced by glucose. Increasing the concentration of glucose promotes dissolution of the crystals and dissociation of the soluble aggregates.

The dose and volume of the subcutaneous or intramuscularly injected depot is similar to that of the ordinary basal insulin preparations, meant to cover basal insulin supply by injection once or twice daily. Inhaled insulin preparations of insulin derivatives having glucose sensor may be taken several times during the day, typically before or during the meals.

Soluble insulin derivatives featuring lipophilic substituents, capable of forming high molecular weight aggregates larger than aldolase (Mw = 158 kDa), have been disclosed (Novo Nordisk, WO 99/21888) the contents of which is hereby incorporated in its entirety by reference. The release of insulin derivative from such aggregates appeared to depend upon diffusion controlled disintegration of the soluble aggregates. However, some high molecular aggregates, formed from selected insulin derivatives, disintegrate and form smaller aggregates when glucose is introduced in the buffer, the higher the glucose concentration the more thorough is the disintegration. Selected insulin analogues, the aggregates of which are especially prone to disintegrate in the presence of glucose, are such in which hydrophobic interactions and hydrogen bonds can form between monomers from different hexamers, *e.g.* by contacts between the OH group of tyrosine B26 in hexamer 1 and an OH group from the substituent introduced on lysine B29 in hexamer 2, and vice versa. The state of aggregation and the power of glucose to diminish this state can be demonstrated by gel filtration in buffers, varying the concentrations of glucose in the eluents. The increased release of insulin derivative from subcutaneous depots can be demonstrated by the different levels of the insulin derivative in the plasma of pigs clamped at various blood glucose levels, *e.g.* 5 and 10 mM, after injection of the

same dose of the insulin derivative. This new concept of glucose dependent insulin release complies with the convenience of the state of the art injection regimens of insulin therapy, and requires neither surgery nor the danger associated with storage of large implanted depots in the body.

Brief description of the drawing

The present invention is further illustrated with reference to the appended drawing wherein

Fig. 1 shows that a faster release of insulin at a high glucose concentration, and a slower release at a low glucose concentration is possible by the multiple interactions between insulin hexamers than by a mechanism involving just one bond.

Detailed description of the Invention

The expression "insulin derivative" as used herein (and related expressions) refers to human insulin or an analogue thereof in which at least one organic substituent is bound to one or more of the amino acids.

By "analogue of human insulin" as used herein (and related expressions) is meant human insulin in which one or more amino acids have been deleted and/or replaced by other amino acids, including non-codeable amino acids, or human insulin comprising additional amino acids, i.e. more than 51 amino acids.

By "depot" is meant the amount of subcutaneous or intramuscularly injected or inhaled insulin preparation, either in the form of crystalline preparations, such as NPH and Lente, or as solutions, such as albumin binding or soluble aggregating or acid solutions of neutral-precipitating, of insulin analogues or insulin derivatives.

By "absorption" is meant the process by which the insulin in the depot is transferred to the circulation.

By "glucose sensor" is meant a chemical group, capable of binding to or reacting with glucose. The glucose sensor is part of the insulin molecule. For reversible binding, the dissociation constant of the sensor-glucose complex is usually in the range from 1 μM to 20 mM. Examples of reversible glucose sensors are organic borates, preferably aryl boronates or other borates, where the attachment to an insulin derivative is via a carbon-boron bond. Alkyl boronates are oxidatively labile and often unstable (Snyder, Kuck and Johnson, J. Am. Chem. Soc 1938, 60, 105). Boronate sensors that bind glucose under physiological conditions are preferred. Simple aryl boronates, such as phenyl boronate,

binds glucose only at relatively high pH, >9 (Shinkai and Takeuchi, Trends Anal. Chem. 1996, 15, 188). Acidic boronates, which bind glucose at physiological pH, are preferred. Examples of such boronate glucose sensors are aminomethyl-aryl-2-boronates (Bielecki, Eggert and Norrild, J. Chem. Soc., Perkin Trans 2 1999, 449), other boronates with amino groups in the vicinity (Shiino et al, J. Controlled Release 1995, 37, 269), or aryl boronates substituted with electron-withdrawing groups (Eggert et al., J. Org. Chem. 1999, 64, 3846), *e.g.* pyridine boronates, pyridinium boronates or sulfo-, carboxy- or nitro-phenyl boronates. These acidic boronates all assume a tetrahedral configuration in aqueous solvent at physiological pH, thereby allowing binding of glucose. Reversible glucose sensors may also be peptides or pseudopeptides, optionally containing boronates. Examples of irreversible glucose binders are oxyamines and hydrazines, which react with glucose to form oximes and hydrazones (Veprek and Jezek, J. Peptide Sci. 1999, 5, 203; Peri, Dumy and Mutter, Tetrahedron 1998, 54, 12269). Examples of useful oxyamine functions are aminoxyacetic acid, AOA (Vilaseca et al. Bioconjugate Chem. 1993, 4, 515), and O-aminoserine, Ams (Spetzler and Hoeg-Jensen, J. Pept. Sci. 1999, 5, 582).

In one preferred embodiment the present invention is based on the discovery of soluble and aggregated forms of insulin derivatives, wherein the state of aggregation is being influenced by glucose. The aggregate is preferably soluble in water at neutral pH, in the range of 6.8 to 8.5. The soluble, aggregated forms of insulin derivatives dissociates slowly after subcutaneous injection, making them suitable for a long-acting insulin preparation, the advantage being that the preparation contains no precipitate. The higher the concentration of glucose is in the tissue the higher the rate of dissociation and of the subsequent absorption. The advantages of soluble rather than suspended preparations are higher precision in dosing, avoidance of shaking of the vial or pen, allowance for a thinner needle meaning less pain during injection, easier filling of vials or cartridge and avoidance of a ball in the cartridge used to suspend the precipitate in the absence of air.

The apparent volume of elution of aggregates, as estimated by the K_{AV} value, changes to a larger value when the glucose concentration is increased from 0 to 20 mM, as determined by gel filtration using a Superose® 6 HR gel. In order to achieve an optimal effect of glucose on the state of aggregation in this experiment, the concentration of sodium chloride should be decreased just to obtain an aggregation about the size of aldolase (i.e. the K_{AV} value of 0.5).

The aggregated form can be observed for insulin derivatives under conditions where the hexameric unit is known to exist for most insulins. Thus, in a preferred embodiment, the aggregated form is composed of hexameric subunits, preferably of at least 4, more preferably 5 to 500, hexameric subunits. Any hexameric subunit of the aggregated forms of this invention may have any of the known R_6, R_3T_3, or T_6 structures (Kaarsholm, Biochemistry 28, 4427-4435, 1989).

Substances like Zn^{2+} known to stabilise the hexameric unit are also found to stabilise the aggregated form of some insulin derivatives. The building blocks forming the aggregates may be the hexameric units known from the X-ray crystallographic determined structure of insulin (Blundell, Diabetes 21 (Suppl. 2), 492-505, 1972). Ions like Zn^{2+}, known to stabilise the hexameric unit as 2 or 4 Zn^{2+}/hexamer complexes (Blundell, Diabetes 21 (Suppl. 2), 492-505, 1972), are essential for the formation of aggregates for most insulin analogues and derivatives. Thus, preparations of glucose dependent aggregating insulin derivatives according to this invention preferably comprises at least 2 zinc ions, more preferably 2 to 5 zinc ions, still more preferably 2 to 3 zinc ions, per 6 molecules of insulin derivative. Moreover, the preparations advantageously comprise at least 3 molecules of a phenolic compound per 6 molecules of insulin derivative. In the central cavity of the 2 Zn^{2+}/hexamer structure 6 residues of Glu^{B13} provide binding sites for up to 3 Ca^{2+} ions (Sudmeier et al., Science 212, 560-562, 1981). Thus, addition of Ca^{2+} ions stabilises the hexamer and may be added to the pharmaceutical compositions, on the condition that the insulin derivative remains in solution.

The disappearance half-time of the aggregate of the invention after subcutaneous injection in healthy human subjects, having normal blood glucose concentrations about 5 mM, is preferably as long as or longer than that of a human insulin NPH preparation.

In a particularly preferred embodiment of the present invention, the aggregate is composed of insulin derivatives, which have an albumin binding which is lower than that of Lys^{B29}(N^{ε}-tetradecanoyl) des(B30) human insulin.

The substituent at the lysine residue of the insulin derivative of the aggregate according to the invention is preferably a lipophilic group containing from 6 to 40 carbon atoms.

More preferred are substituents capable of forming hydrogen bonds to same substituent situated in an adjacent monomer of another hexamer, or to the OH group of tyrosine B26 in an adjacent monomer of another hexamer.

In a preferred embodiment the lipophilic substituent is 5-α lithocholic acid, 5-β lithocholic acid, the 5-α or 5-β isomers of cholic acid, hyocholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, hyodeoxycholic acid or cholanic acid.

In another preferred embodiment the lipophilic substituent is connected to a lysine residue using an amino acid linker. According to this embodiment the lipophilic substituent is advantageously connected to a lysine residue via a γ- or an α-glutamyl linker or via a β- or an α-aspartyl linker.

In yet another preferred embodiment the lipophilic substituent comprises the glucose sensor in the form of a borate group, an aryl boronate, an amino aryl boronate, an oxyamine, a hydrazine or a glucose binding peptide.

The present invention furthermore provides novel insulin derivatives capable of forming aggregates, in which the degree of aggregation is conversely correlated to the glucose concentration. These insulin derivatives may be provided in the form of aggregates in pharmaceutical preparations or, alternatively, they may be provided in a non-aggregated form in pharmaceutical preparations, in which case the aggregates form after subcutaneous injection of said preparations.

Accordingly, the present invention furthermore is concerned with pharmaceutical preparations comprising an aggregate of insulin derivatives or non-aggregated insulin derivatives, which form aggregates after subcutaneous injection, the degree of aggregation being conversely correlated to the glucose concentration. The dissociation of the soluble insulin polymers into soluble insulin hexamers by the action of glucose molecules can be described by the following equation:

$$\text{insulin polymer} + n\ \text{glucose} \leftrightarrow \text{insulin hexamer}$$

where n is the number of glucose molecules required to break the polymeric insulin network, releasing the insulin hexamers from the network. The advantage of n being larger than 1 is apparent from Fig. 1, which shows that increasing n from 1 to 6 increases the steepness of the curve for the fraction of free insulin hexamers over polymer, bound insulin hexamers. Thus, a faster release of insulin at a high glucose concentration, and a slower release at a low glucose concentration, is possible by the multiple interactions between insulin hexamers than by a mechanism involving just one bond.

Preferably, the pharmaceutical composition according to the present invention comprises aggregates, a substantial fraction of which have a higher molecular weight than aldolase as determined by gel filtration using the medium of the composition as eluent.

In another embodiment, a pharmaceutical composition comprises both aggregating and rapid acting insulin analogues, the latter preferably being human insulin or one of the insulin analogues Asp^{B28} human insulin, $Lys^{B28}Pro^{B29}$ human insulin, $Gly^{A21},Lys^{B3},Ile^{B28}$ human insulin or des(B30) human insulin. Such a preparation will provide both a rapid

onset of action as well as a prolonged action profil , the latter being under influence of the glucose concentration of the diabetic patient. In the case the two insulins of the mixture form mixed hexamers both will be under influence of the glucose concentration

In this embodiment, the pharmaceutical preparation preferably comprises aggregating insulin and rapid acting insulin in a molar ratio of 90:10 to 10:90.

The slow dissociation of the aggregated forms may be further slowed down in vivo by the addition of physiological acceptable agents that increase the viscosity of the pharmaceutical preparation. Thus, the pharmaceutical preparation according to the invention may furthermore comprise an agent that increases the viscosity, preferably polyethylene glycol, polypropylene glycol, copolymers thereof, dextrans and/or polylactides.

In yet another embodiment the insulin derivative containing a glucose sensing group is prepared as a crystalline NPH preparation, using protamine to form the crystals, or as a crystalline Lente preparation, using Zn^{2+}-Ions in the crystals. In these cases the rate of dissolution of the crystals is enhanced by the interaction between glucose and the glucose sensing group.

In yet another embodiment the protracted insulin compositions are solutions having a pH value below physiological pH from which the insulin analogue will precipitate because of the rise in the pH value when the solution has been injected. Such analogues are described in EP 0 254 516 B1 (Novo) and EP 0 368 187 B1 (Hoechst). These analogues have mutated the asparagine in position A21 to an acid-stabile residue such as glycine, serine or alanine and have mutations to increase the net charge of the molecule by about 2, *e.g.* by B27 Thr -> Arg and B30 Thr-OH -> Thr-NH_2 or have additional basic residues, *e.g.* B31-B32 Arg-Arg. When this principle is combined with the present invention by incorporation of the glucose-sensor in these insulin analogues, the solubility of the crystals is enhanced by the interaction between glucose and the glucose sensing group, facilitating the absorption.

Sites enabling the attachment of a glucose sensor are the N-terminal amino groups of A1 glycine and B1 phenylalanine and the ε-amino group of B29 lysine. One or more additional lysine residues may be incorporated for this purpose, *e.g.* in position B3. Furthermore the glucose sensor may be incorporated as part of the peptide chain, preferably in the C-terminal part of the B-chain.

The pharmaceutical preparation preferably further comprises a buffer substance, such as a TRIS, phosphate, glycine or glycylglycine buffer, an isotonicity agent, such as sodium chloride, glycerol, mannitol and/or lactose, and phenol and/or m-cresol as preservatives. Among the auxiliary substances of a pharmaceutical preparation the sodium

chloride, used as isotonic agent, zinc- and calcium-ions, promoting and stabilizing the formation of hexamers, are particular important by facilitating the aggregation in the preparation and thereby effectively prolong the time of disappearance from the site of injection. The pharmaceutical composition according to the invention preferably comprises Cl^- -ions in a concentration of 5 to 150 mM.

The most preferred pharmaceutical preparation is a preparation containing 0.1-2 mM of an insulin derivative according to the present invention, 0.3-0.9% Zn (w/w relative to insulin derivative), and phenolic compounds like phenol or m-cresol or mixtures hereof in a total concentration of 5-50 mM, and Cl^- -ions in a concentration of 10 mM to 100 mM.

The present invention furthermore relates to a method of treating diabetes mellitus comprising administering to a person in need of such treatment an effective amount of water-soluble aggregates of insulin derivatives according to the invention or effective amount an insulin derivative according to the invention, capable of forming water-soluble aggregates upon subcutaneous injection, aggregate size depending on the glucose concentration.

The insulin derivatives of the invention can be prepared by the general methods disclosed in WO 95/07931 (Novo Nordisk A/S), WO 96/00107 (Novo Nordisk A/S), WO 97/31022 (Novo Nordisk A/S), PCT application No. DK97/00296 (Novo Nordisk A/S), EP 511 600 (Kuraray Co. Ltd.) and EP 712 862 (Eli Lilly).

Some of the derivatives listed in the aforementioned patent applications, and described in the publications of Markussen, Diabetologia 39, 281-288, 1996; Kurzhals, Biochem J. 312, 725-731, 1995; Kurzhals, J. Pharm Sciences 85, 304-308, 1996; and Whittingham, Biochemistry 36, 2826-2831, 1997 as being protracted due to the albumin binding mechanism, do also posses the ability to form high molecular weight soluble aggregates. Lys^{B29}(N^{ε}-lithocholyl-γ-glutamyl) des(B30) human insulin from WO 95/07931 and Lys^{B29}(N^{ε} ω-carboxyheptadecanoyl) des(B30) human insulin from WO 97/31022 are examples of insulin derivatives capable of forming high molecular weight soluble aggregates at neutral pH.

DETERMINATION OF AGGREGATE FORMATION

The aggregated form is demonstrated by gel filtration using a gel with an exclusion limit of 5000 kDa for globular proteins. An aqueous buffer system at neutral pH is used in the gel filtration and the insulin derivatives in the aggregated state are applied to the column in the form of a pharmaceutical preparation at a concentration of 600 nmol insulin/ml. The aggregated states of the insulin derivatives elute before aldolase, which has a molecular weight of 158 kDa.

The gel filtration experiment using the conditions prescribed in this section is the direct physicochemical method to reveal the potential aggregate formation property of an insulin derivative. Disappearance after subcutaneous injection in pigs reflects the combination of the polymer formation, glucose concentration and albumin binding properties of the insulin derivative, besides a variety of biological factors.

The formation of glucose-dependent, high molecular weight soluble aggregates is demonstrated by gel filtration using a column of Superose® 6 HR in an aqueous neutral eluent comprising from 20 to 140 mM sodium chloride, 10 mM trishydroxymethylaminomethan at pH 7.4 and glucose concentration varying from 0 to 20 mM. This buffer system was chosen to mimic the pH of the tissue, in order to be able to detect derivatives changing their state of aggregation under conditions similar to those after the subcutaneous injection. In other buffer systems having too low a concentration of sodium chloride, or a lower or higher pH value, the derivatives may not appear in the aggregated state.

Gel filtration assay for aggregation: A gel filtration column useable for separation of a wide molecular weight range, Superose 6 HR 10/30 (Pharmacia Biotech), is eluted at 37 °C by sodium chloride 140 mM, trishydroxymethylaminomethan 10 mM, and hydrochloric acid added to pH 7.4. A run time of 90 min. (0.25 ml/min.) corresponding to the total column volume is followed by a washing period of about 3 column volumes (0.5 ml/min.). The injection volume was 200 µl or about 1 % of the total column volume. For insulin derivatives eluting partly after the column volume the gel filtration is repeated with a diluted eluent (one third of water and next two third of water). The aggregated states of the insulin derivatives elute before aldolase, which has a molecular weight of 158 kDa. The dissociation effect of glucose on the state of aggregation is tested by inclusion of 5, 10 or 20 mM glucose in the eluent.

Alternative methods to study the state of aggregation are light scattering, osmometry and ultracentrifugation.

EXAMPLES

Example 1

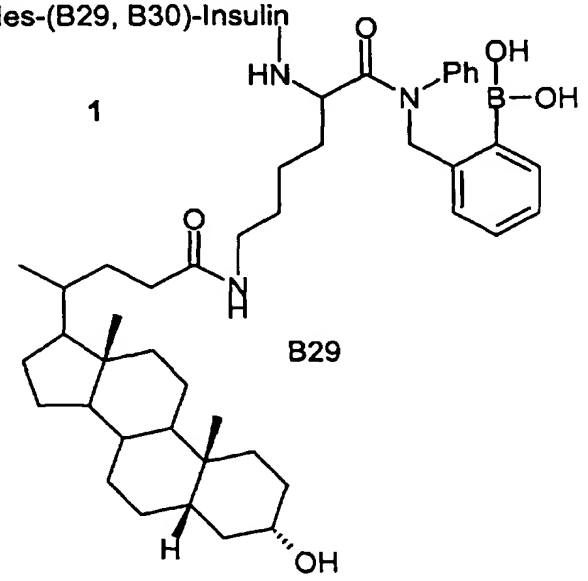
$Lys^{B29}(N^{\varepsilon}$-lithocholoyl)-N-phenyl-B29-benzylamide-2-boronic acid, des(B30) insulin, **1**.

2,2-dimethylpropane-1,3-diyl-2-(bromomethyl)phenylboronate (Bielecki, Eggert and Norrild, J. Chem. Soc. Perkin Trans 2, 1999, 449) was reacted with aniline to give N-phenyl-benzylamine-2-(2,2-dimethylpropane-1,3-diyl)boronate. This amine was coupled to insulin LysB29 using achromobacter

lyticus protease (Morihara and Ueno, Biotech. Bioeng. 1991, 37, 693). LysB29 was subsequently acylated selectively using N-hydroxysuccinimidyl lithocholate (US5646242) to give structure **1**.

des-(B29, B30)-Insulin

1



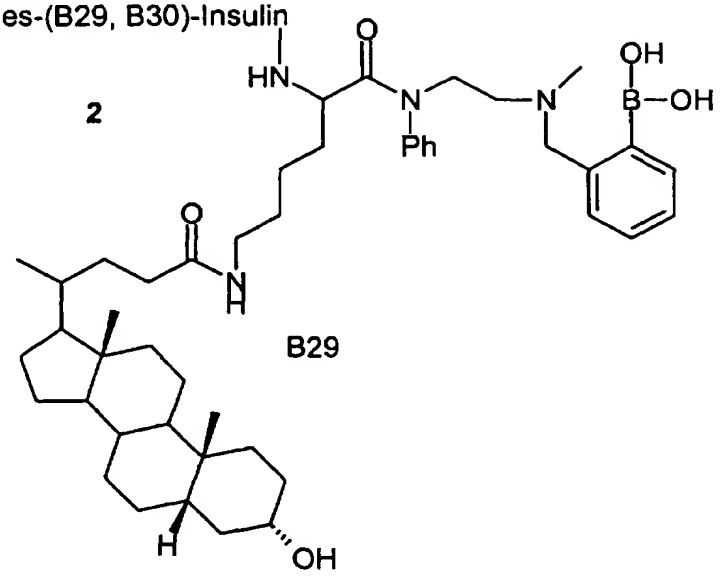
In the above procedure, lithocholic acid can be substituted with other lipophilic groups, preferably hyocholic acid, hyodeoxycholic acid or chenodeoxycholic acid.

Example 2

$Lys^{B29}(N^{\varepsilon}$-lithocholoyl)-N'-methyl-N'-(benzyl-2-boronic acid)-2-amino-N-phenyl-B29-ethylamide, des(B30) insulin, **2**.

2,2-dimethylpropane-1,3-diyl-2-(bromomethyl)phenylboronate (Bielecki, Eggert and Norrild, J. Chem. Soc. Perkin Trans 2, 1999, 449) was reacted with N'-phenyl-N-methylethylendiamine to give N'-phenyl-N-methyl-N-benzyl-2-(2,2-dimethylpropane-1,3-diyl)boronate ethylenediamine. This amine was coupled to insulin LysB29 using achromobacter lyticus protease (Morihara and Ueno, Biotech. Bioeng. 1991, 37, 693). LysB29 was subsequently acylated selectively using N-hydroxysuccinimidyl lithocholate (US5646242) to give structure **2**.

des-(B29, B30)-Insulin

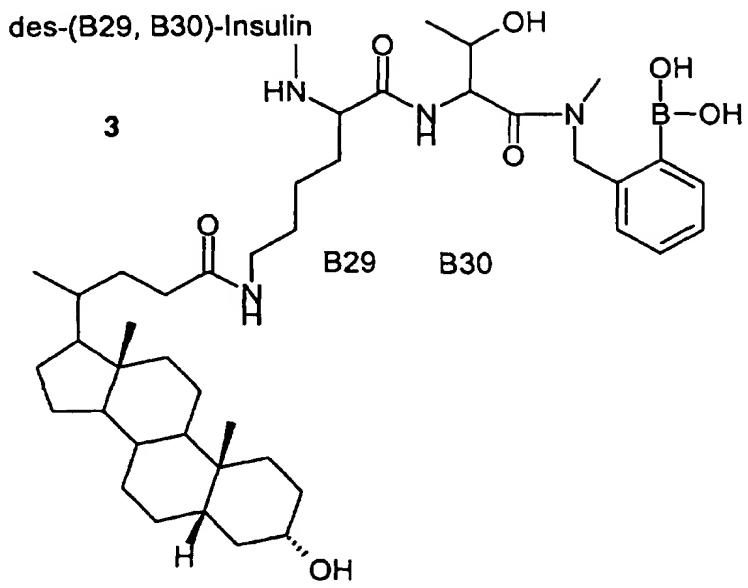


In the above procedure, lithocholic acid can be substituted with other lipophilic groups, preferably hyocholic acid, hyodeoxycholic acid or chenodeoxycholic acid.

Example 3

$Lys^{B29}(N^{\varepsilon}$-lithocholoyl)-N-phenyl-B30-(benzylamide-2-boronic acid) insulin, **3**.

2,2-dimethylpropane-1,3-diyl-2-(bromomethyl)phenylboronate (Bielecki, Eggert and Norrild, J. Chem. Soc. Perkin Trans 2, 1999, 449) was reacted with methylamine to give N-methyl-benzylamine-2-(2,2-dimethylpropane-1,3-diyl)boronate. This amine was coupled to tert-butyloxycarbonyl-threonine (Boc-Thr) using dicyclohexylcarbodimide and 1-hydroxybenzotriazole and the Boc-group was removed with trifluoroacetic acid. The resulting threonine N-methyl-benzylamide-2-boronate was coupled to insulin LysB29 using achromobacter lyticus protease (Morihara and Ueno, Biotech. Bioeng. 1991, 37, 693). LysB29 was subsequently acylated selectively using N-hydroxysuccinimidyl lithocholate (US5646242) to give structure **3**.



In the above procedure, lithocholic acid can be substituted with other lipophilic groups, preferably hyocholic acid, hyodeoxycholic acid or chenodeoxycholic acid.

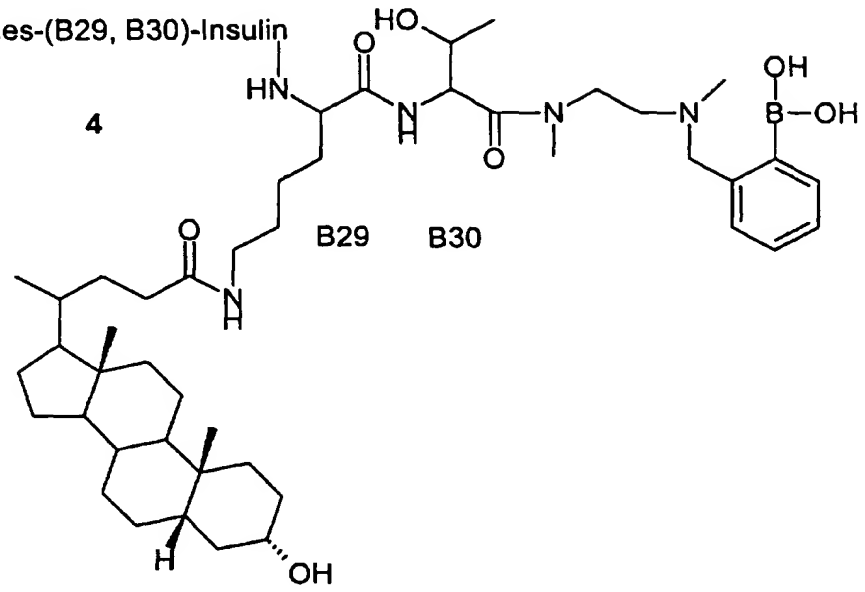
Example 4

$Lys^{B29}(N^{\varepsilon}$-lithocholoyl)-N'-methyl-N'-(benzyl-2-boronic acid)-2-amino-N-methyl-B30-ethylamide insulin, **4.**

2,2-dimethylpropane-1,3-diyl-2-(bromomethyl)phenylboronate (Bielecki, Eggert and Norrild, J. Chem. Soc. Perkin Trans 2, 1999, 449) was reacted with N',N-dimethylethylendiamine to give N'-methyl-N-methyl-N-benzyl-2-(2,2-dimethylpropane-1,3-diyl)boronate. This amine was coupled to tert-butyloxycarbonyl-threonine using dicyclohexylcarbodimide and 1-hydroxybenzotriazole and the Boc-group was removed with trifluoroacetic acid. The resulting threonine N-methyl-N'-methyl-N'-benzyl-(2-(2,2-dimethylpropane-1,3-diyl)boronate) 2-amino-ethylamide was coupled to insulin LysB29 using achromobacter lyticus protease (Morihara and Ueno, Biotech. Bioeng. 1991, 37, 693). LysB29 was subsequently acylated selectively using N-hydroxysuccinimidyl lithocholate (US5646242) to give structure **4.**

des-(B29, B30)-Insulin

4

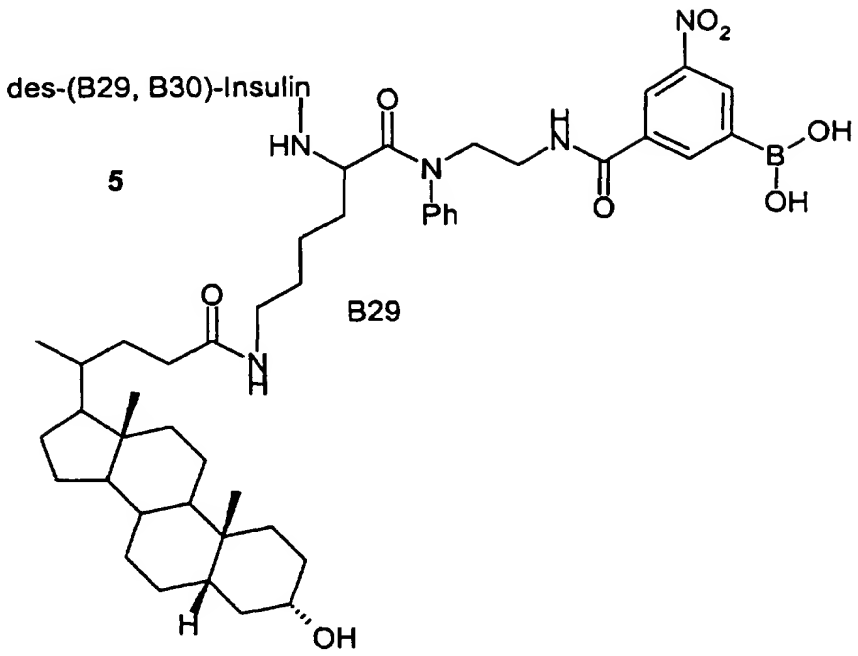


In the above procedure, lithocholic acid can be substituted with other lipophilic groups, preferably hyocholic acid, hyodeoxycholic acid or chenodeoxycholic acid.

Example 5

$Lys^{B29}(N^{\varepsilon}$-lithocholoyl)-N'-(benzoyl-3-borno-5-nitro)-2-amino-N-phenyl-B30-ethylamide, des(B30) insulin, **5.**

3-borono-5-nitro-benzoic acid (Combi-Blocks, San Diego, CA, USA) was coupled to N-phenyl ethylenediamine using dicyclohexylcarbodimide and 1-hydroxybenzotriazole. The resulting N'-(3-borono-5-nitro-benzoyl) N-phenyl ethylenediamine was coupled to insulin LysB29 using achromobacter lyticus protease (Morihara and Ueno, Biotech. Bioeng. 1991, 37, 693). LysB29 was subsequently acylated selectively using N-hydroxysuccinimidyl lithocholate (US5646242) to give structure **5**.

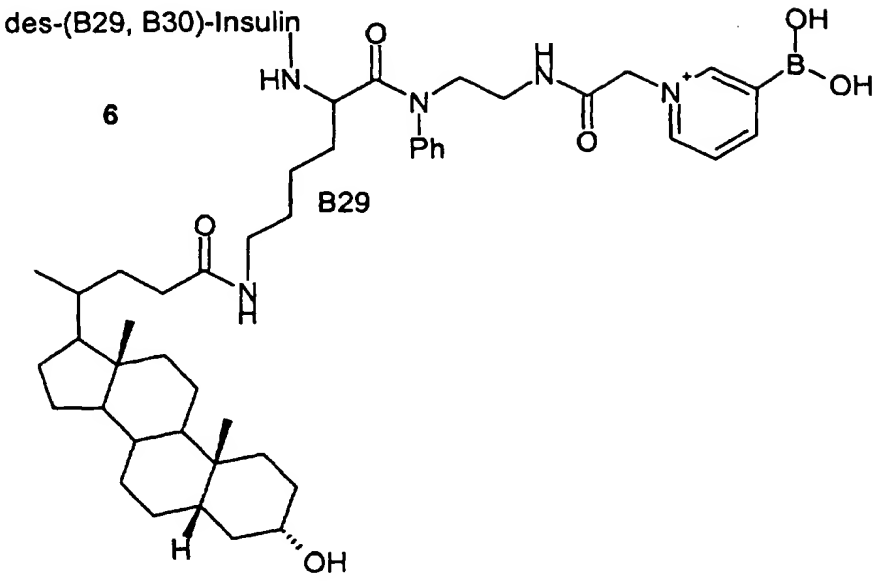


In the above procedure, lithocholic acid can be substituted with other lipophilic groups, preferably hyocholic acid, hyodeoxycholic acid or chenodeoxycholic acid.

Example 6

$Lys^{B29}(N^{\varepsilon}$-lithocholoyl)-2-(pyridinium-3-boronic acid)-acetyl-2-amino-N-phenyl-B30-ethylamide, des(B30) insulin, **6.**

2,2-dimethylpropane-1,3-diyl-3-borono-pyridine (Eggert, Frederiksen, Morin and Norrild, J. Org. Chem. 1999, 64, 3846) was reacted with bromoacetic acid. The resulting 2-(2,2-dimethylpropane-1,3-diyl-3-borono pyridinium) acetic acid was coupled to N-phenyl ethylenediamine using dicyclohexylcarbodimide and 1-hydroxybenzotriazole. The resulting amine was coupled to insulin LysB29 using achromobacter lyticus protease (Morihara and Ueno, Biotech. Bioeng. 1991, 37, 693). LysB29 was subsequently acylated selectively using N-hydroxysuccinimidyl lithocholate (US5646242) to give structure **6.**

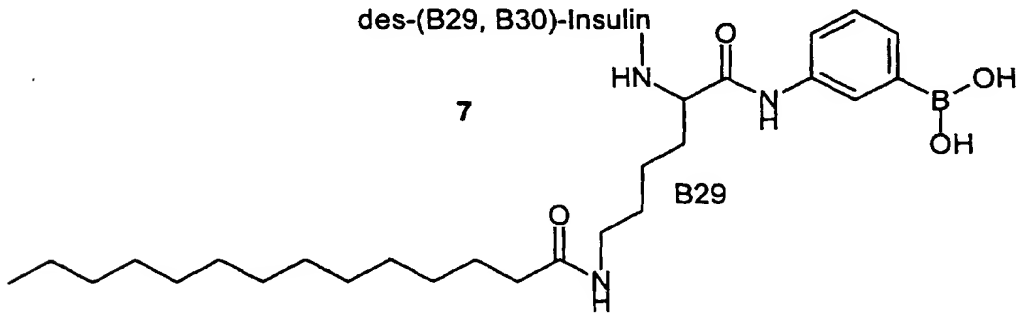


In the above procedure, lithocholic acid can be substituted with other lipophilic groups, preferably hyocholic acid, hyodeoxycholic acid or chenodeoxycholic acid.

Example 7

$Lys^{B29}(N^{\varepsilon}$-tetradecanoyl)-B29-anilide-3-boronic acid, des(B30) insulin, **7**.

Aniline-3-boronic acid was coupled to insulin LysB29 using achromobacter lyticus protease (Morihara and Ueno, Biotech. Bioeng. 1991, 37, 693). LysB29 was subsequently acylated selectively using N-hydroxysuccinimidyl tetradecanate (US5646242) to give structure **7**.

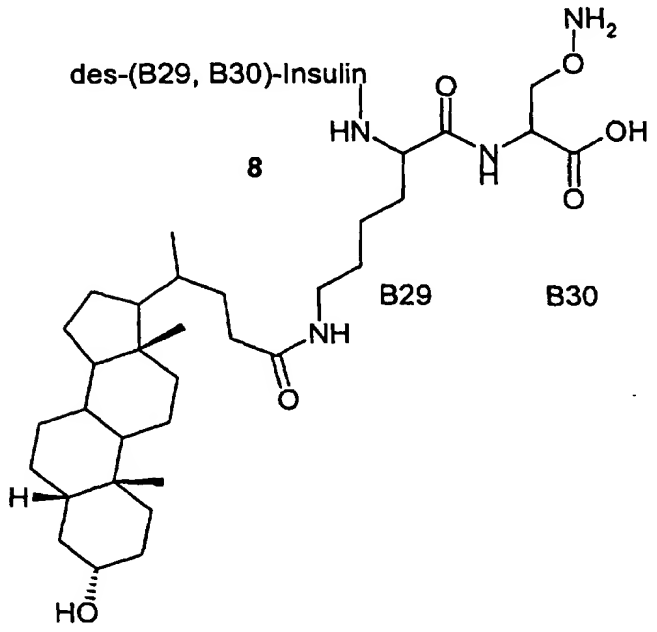


In the above procedure, tetradecanoic acid can be substituted with other lipophilic groups, preferably lithocholic acid, hyocholic acid, hyodeoxycholic acid or chenodeoxycholic acid.

Example 8

$Lys^{B29}(N^{\varepsilon}$-lithocholoyl)-$Ams^{B30}$ insulin, **8.**

Ams(Boc)-OBu^t (Spetzler and Hoeg-Jensen, J. Pept. Sci. 1999, 5, 582) was coupled to insulin LysB29 using achromobacter lyticus protease (Morihara and Ueno, Biotech. Bioeng. 1991, 37, 693). LysB29 was subsequently acylated selectively using N-hydroxysuccinimidyl lithocholate (US5646242) and the insulin was deprotected with trifluoroacetic acid to give structure **8.**



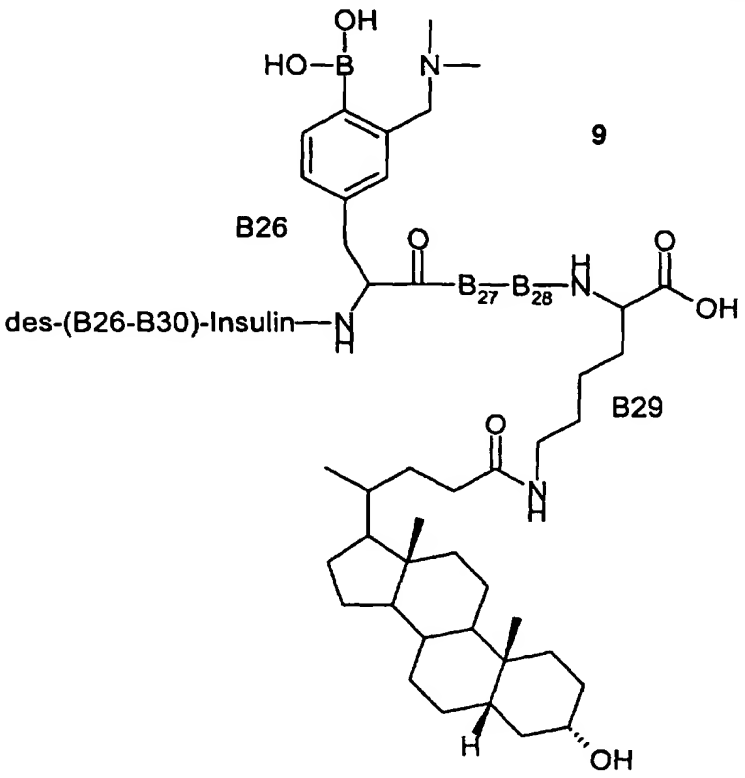
In the above procedure, lithocholic acid can be substituted with other lipophilic groups, preferably hyocholic acid, hyodeoxycholic acid or chenodeoxycholic acid.

Example 9

Phe^{B26}(3-(N,N-dimethyl-aminomethyl)-4-boronic acid), $Lys^{B29}(N^{\varepsilon}$-lithocholoyl) des(B30) insulin, **9.**

3-(N,N-dimethyl-aminomethyl)-4-borono-phenylalanine (NBPhe) was made from 4-boronophenylalanine (RSP, Worchester, MA, USA) and incorporated into the following peptide se-

quence using standard solid-phase peptide synthesis, Gly-Phe-Phe-NBPhe-Thr-Pro-Lys(lithocholoyl). This peptide was coupled to des-octapeptide insulin using trypsin.



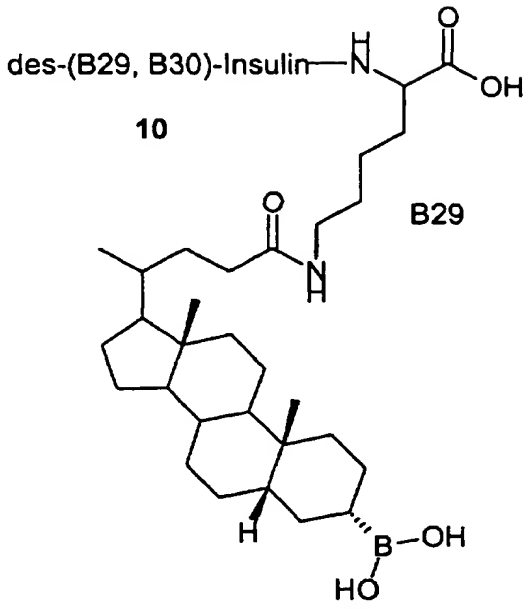
9

In the above procedure, lithocholic acid can be substituted with other lipophilic groups, preferably hyocholic acid, hyodeoxycholic acid or chenodeoxycholic acid.

Example 10

$Lys^{B29}(N^{\varepsilon}$-cholanoyl-3-boronic acid) des(B30) insulin, **10.**

3-borono-cholanoyl was made from lithocholic acid by elimination (Templeton et al. Steroids 2000, 65, 219) and hydroboration (Kirk et al. J. Chem. Soc. Perkin Trans 1 1976, 1836). The lithocholate was converted to its N-hydroxysuccinimidyl ester and selectively acylated onto insulin LysB29 (US5646242).

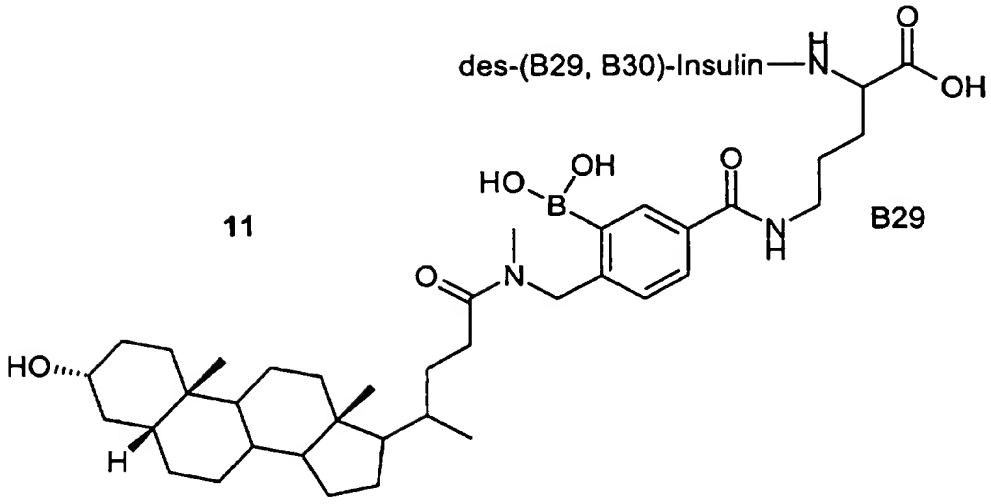


In the above procedure, cholanoyl can be substituted with other lipophilic groups, preferably 6,7-dihydroxycholanoyl, 6-hydroxycholanoyl or 7-hydroxycholanoyl.

Example 11

$Lys^{B29}(N^{\varepsilon}$-(lithocholoyl-(N-methyl-4-aminomethyl-3-boronic acid-benzoyl))), des(B30) insulin, **11.**

N-methyl-4-aminomethyl-3-borono-benzoic acid (Combi-Blocks, San Diego, CA, USA) was acylated using N-hydroxysuccinimidyl lithocholate. The lithocholyl benzoic acid was converted to its N-hydroxysuccinimidyl ester and coupled selectively to insulin LysB29 (US5646242) to give 2,2-dimethylpropane-1,3-diyl-2-(bromomethyl)phenylboronate (Bielecki, Eggert and Norrild, J. Chem. Soc. Perkin Trans 2, 1999, 449) was reacted with aniline to give N-phenyl-benzylamine-2-(2,2-dimethylpropane-1,3-diyl)boronate. This amine was coupled to insulin LysB29 using achromobacter lyticus protease (Morihara and Ueno, Biotech. Bioeng. 1991, 37, 693). LysB29 was subsequently acylated selectively using N-hydroxysuccinimidyl lithocholate (US5646242) to give structure **11**.



In the above procedure, lithocholic acid can be substituted with other lipophilic groups, preferably hyocholic acid, hyodeoxycholic acid or chenodeoxycholic acid.

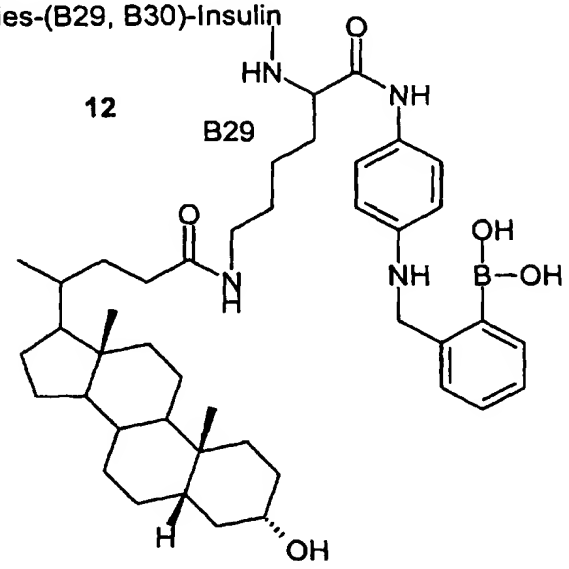
Example 12

$Lys^{B29}(N^{\varepsilon}$-Lithocholoyl)-4-N-(benzyl-2-boronic acid)-4-amine-B29-anilide, des(B30) insulin, **12.**

2,2-dimethylpropane-1,3-diyl-2-(bromomethyl)phenylboronate (Bielecki, Eggert and Norrild, J. Chem. Soc. Perkin Trans 2, 1999, 449) was reacted with 1,4-phenylenediamine to give 1,4-phenylenediamine-N-benzylamine-2-(2,2-dimethylpropane-1,3-diyl)boronate. This amine was coupled to insulin LysB29 using achromobacter lyticus protease (Morihara and Ueno, Biotech. Bioeng. 1991, 37, 693). LysB29 was subsequently acylated selectively using N-hydroxysuccinimidyl lithocholate (US5646242) to give structure **12.**

des-(B29, B30)-Insulin

12



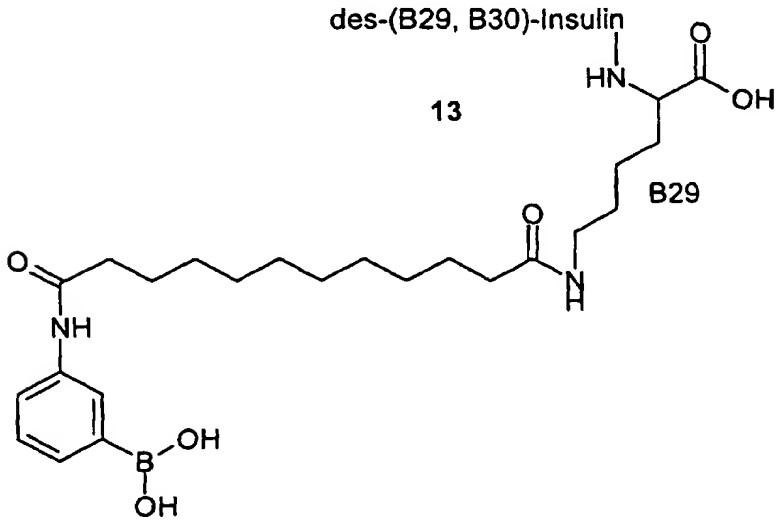
In the above procedure, lithocholic acid can be substituted with other lipophilic groups, preferably hyocholic acid, hyodeoxycholic acid or chenodeoxycholic acid.

Example 13

$Lys^{B29}(N^{\varepsilon}$-(ω-carboxamidophenyl-3-boronic acid nonadecanoyl)) des(B30) insulin **13.**

des-(B29, B30)-Insulin

13



The mono hydroxysuccinimidyl ester of dodecandicarboxylic was reacted with 3-borono-aniline. The resulting ω-carboxamidophenyl-3-boronic acid nonadecanoyl was converted to its hydroxysuccinimidyl ester and acylated selectively onto insulin LysB29 (US5646242).

Example 14

Synthesis of N-succinimidyl *tert*-butyloxycarbonylaminoxy acetate, Boc-AOA-OSu.

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tert-Butyloxycarbonylaminoxy acetic acid was dissolved in ice-cooled ethyl acetate or acetonitrile and treated with N,N'-dicyclohexylcarbodiimide (1.0 equivalent) and N-hydroxysuccinimide (1.0 equivalent). The reaction mixture was stirred at room temperature overnight. The generated N,N'-dicyclohexylurea was removed by filtration and the solvent was removed in vacuo. The crude Boc-AOA-OSu was either used directly in the next step or purified by recrystallisation from chloroform. Adopted from Kurth et al. J. Med. Chem. 1993, 36, 1255.

Example 15

Synthesis of Lys^{B29}(AOA) des(B30) insulin **15**.

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1 g of des(B30) human insulin was dissolved in 50 ml 0.05 M boric acid by adjusting the pH to 10.2 by 1 N NaOH and placed in a thermostat at 15°C. To the solution was added 61 mg of Boc-AOA-Osu dissolved in 50 ml acetonitrile. The reaction is stopped after 1 h by addition of 19 ml 0.2 N ethanolamine, pH 9.0. The product was precipitated by addition of water to a total volume of 250 ml, adjusting the pH to 5.5 by HCl and cooling the solution to -20°C. The precipitate was isolated by centrifugation at -10°C and dried *in vacuo*. Mass spectrometry revealed the parent insulin compound, the monoacylated insulin, and diacylated insulin. The dried product was treated for 1 h at room temperature with 10 ml trifluoroacetic acid plus 0.3 ml triisopropylsilane. The reaction mixture was dripped into 100 ml of cold diethyl ether; the formed precipitate was isolated and dried *in vacuo*. Finally, the compound **15** was purified by RP-HPLC at pH 4.0 using a gradient from 20 to 60% ethanol.

Mw: theory 5780; found 5778 by MALDI.

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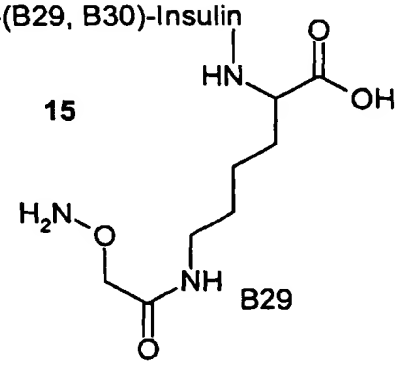
des-(B29, B30)-Insulin



Example 16

Crystalline protamine preparation of Lys^{B29}(AOA) des(B30 insulin.

1. Stock solution of Lys^{B29}(AOA) des(B30 insulin. 35.0 mg Lys^{B29}(AOA) des(B30 insulin was dissolved in water by addition of 32 µl 1 N HCl, 375 µl m-cresol solution (20 mg/ml), 65 µl phenol solution (50 mg/ml), 80 mg glycerol, and 32.7 µl $ZnCl_2$ solution (10 mg/ml), finally adjusting the volume to 5 ml. The pH is about 3.

2. 2.25 ml of this stock solution is mixed with 197 µl of a 10 mg/ml solution in water of protamine sulfate.

3. To the mixture is added 2.25 ml of a phosphate pH 8.0 buffer comprising 375 µl m-cresol solution (20 mg/ml), 65 µl phenol solution (50 mg/ml), 80 mg glycerol, 1.85 ml 70 mM Na_2PO_4, and the volume adjusted to 5 ml with water. The pH of the second mixture is about 5-6, and an amorphous precipitate of Lys^{B29}(AOA) des(B30 insulin and protamine is formed. The pH is adjusted to 7.3 by 1 N NaOH. Crystals of Lys^{B29}(AOA) des(B30 insulin-protamine appeared during storage at room temperature.

CLAIMS

1. An insulin derivative containing a glucose-sensing group.
2. An insulin derivative according to claim 1, the glucose-sensing group being an aryl boronate derivative, optionally having an amino group in proximity to the borate moiety.
3. An insulin derivative according to claim 2, the glucose sensing aryl boronate being located in position 26 of the B-chain of insulin.
4. An insulin derivative according to claim 1, the glucose sensing group being a peptide or pseudopeptide, optionally comprising Asn, Trp, His, Asp, Arg or a boronate containing amino acid in the sequence.
5. An insulin derivative according to claim 1, the glucose-sensing group being a hydrazine or an oxamine.
6. An insulin derivative according to claim 2, 4 or 5, the glucose sensing peptide being comprised within the residues 26-30 of the B-chain, optionally extended beyond the C-terminal residue 30 of the B-chain.
7. An insulin derivative according to claim 1, the glucose-sensing group being built into a substituent capable of forming high molecular aggregates.
8. An insulin derivative according to claim 7, the glucose-sensing group being an aryl boronate and the substituent causing aggregation being a hydrophobic group.
9. An insulin derivative according to claim 8, the hydrophobic group being a derivative of a bile acid selected from the group comprising lithocholic acid, hyocholic acid, hyodeoxycholic acid and chenodeoxycholic acid.
10. An insulin derivative according to claim 8, the hydrophobic group being a derivative of an α,ω- dicarboxylic acid having from 10 to 30 C-atoms.
11. An insulin analogue derivatised by a carbohydrate (monosaccharide, disaccharide, trisaccaride) or a poly-ol, capable of binding to an insulin analogue derivatised by a glucose-sensing group.
12. An insulin derivative according to claims 1-10, having a carbohydrate or a poly-ol substitution in addition to the glucose-sensing group.
13. An insulin derivative or mixture of derivatives according to claims 1-12, capable of forming soluble, high molecular aggregates, molecular weight > 150 kDa.

14. A water soluble, protracted, glucose dependent pharmaceutical composition comprising an insulin derivative or a mixture of insulin derivatives according to claims 1-10 with insulin analogues according to claim 11.

15. A soluble, long-acting, glucose dependent insulin release, insulin preparation of an insulin analogue or mixtures of insulin analogues according to claims 1-13.

16. A soluble, biphasic-acting insulin preparation comprising an insulin analogue according to claims 1-12 mixed with human insulin or an analogue with rapid onset of action, such as human insulin or des(B30) human insulin or Asp^{B28} human insulin or $Lys^{B28}Pro^{B29}$ human insulin or $Gly^{A21},Lys^{B3},Ile^{B28}$ human insulin, in ratios from 10:1 to 1:10.

17. A soluble insulin preparation characterized by having a rate of absorption from an injected depot, which increases as the glucose concentration in the tissue increases, and decreases as the glucose concentration decreases.

18. Crystalline preparations of insulin derivatives according to claims1-12.

NOVO NORDISK A/S

Fig. 1.